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RECENT DEVELOPMENTS IN THE APPLICATION OF X-RAY MICROANALYSIS TO THE STUDY OF FOOD SYSTEMS

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Abstract

Low temperature scanning electron microscopy in conjunction with X-ray microanalysis can be used to study not only the internal structure of intact bulk food systems but also the distribution of their constitutive chemical elements. However, the considerable practical problems that are usually encountered when performing X-ray analysis on frozen samples include: a) the controlled deposition of a good quality carbon film to prevent charging, b) producing digital elemental distribution maps of elements whose X-ray spectral energy peaks partially or completely overlap and c) controlling the plane of fracture through the specimen and ensuring that the resulting topography allows meaningful analysis to be performed.

The quality and control of carbon film deposition is greatly improved by the inclusion of a turbomolecular pump in the vacuum system and by using thickness monitoring of the carbon film during deposition. However, the complications of energy peak overlap in digital X-ray mapping can only be overcome by using a procedure which produces maps corrected for all of the spectrum processing routines normally available in quantitative programmes. Another advantage of this approach is that statistics such as FIT index (see appendix) and standard deviation are available for each pixel.

Problems associated with fracturing are avoided if the internal structure of materials is revealed using cryo-milling. This procedure uses a rotating diamond cutting tool to produce a very flat surface on the frozen specimen which is ideal for X-ray analysis and for image analysis of structural components. Quantitative X-ray mapping of milled cocoa beans shows that this preparation procedure does not cause smearing of the chemical components across the surface of the specimen.

Key Words: Food; X-ray analysis; scanning electron microscopy; cocoa bean; carbon coating; cryo-milling; quantitative mapping; chocolate; turbomolecular pump.

Introduction

The application of low temperature scanning electron microscopy (cryo-SEM) to the study of foods and food related systems is now becoming widely established (e.g. Schmidt et al., 1979; Schmidt & van Hooydonk, 1980; Heertje et al., 1987; Brooker, 1988). Its value is that it allows the examination of bulk samples without the removal of fat, water and other volatile components which so often form important structural elements in foods. For example, such diverse foods as dairy cream, margarines and chocolate contain high levels of fat which are invariably lost or altered by normal preparation methods, but which are faithfully preserved by freezing.

In practice, the maximum size of the specimen to be frozen is influenced by its free water content and by the rate of cooling that is necessary to produce acceptably small ice crystals; Robards & Sleytr (1985) have discussed at length the various freezing techniques available and their relative merits. However, it is fortunate that many raw and processed food materials contain either little or no free water or effective cryoprotectants. Thus, confectionery products generally contain low levels of free water in the presence of high concentrations of sucrose whilst in baked products, water is effectively bound by the cereal (and other) proteins.

A considerable advantage of this general approach to specimen preparation is that it permits the study of dynamic changes in complex processes; by freezing samples from sequential stages or by freezing at various time intervals, a complete sequence of structural events can be reconstructed using cryo-SEM. This is especially useful when intermediate stages in structure development are too labile to be preserved by conventional methods of chemical fixation (Brooker et al., 1986).

Because freezing preserves the chemical as well as the structural integrity of foods, X-ray microanalysis can also be used to provide information on the quantity and distribution of specific chemical elements in bulk samples. In the case of highly hydrated food materials, the redistribution of solutes caused by ice crystal formation is a problem only when the size of the

ice crystals is significantly larger than the spatial resolution required from the X-ray analysis; it is worthwhile remembering that the extreme limits of spatial resolution are not always needed.

Although the ability to perform meaningful X-ray analysis has often been cited as a major advantage of cryo-SEM, relatively few papers describing its application to food systems have been published and some of the reasons for this have been discussed briefly elsewhere (Brooker, 1989). Not the least of these is the fact that whereas commercial cryo-preparation units are designed primarily to sputter coat specimens with gold, their facility for the high quality carbon coating required for X-ray analysis is far less adequate. Moreover, in order to get meaningful and reproducible results from quantitative X-ray analysis and digital X-ray mapping, it is important that the surface of the sample is prepared in such a way that it is reasonably flat. Although surface roughness can be accomodated within certain limits during analysis using the peak to background ratio method (Boekestein et al., 1980; Marshall, 1980), freezing and fracturing often results in fracture planes with extremes of topography. This can present serious problems (Hess, 1980) and may necessitate tilting the sample through large angles or selecting only the smooth parts of the surface for analysis, both of which are undesirable. These problems, together with consideration of the application of the new quantitative X-ray mapping techniques, are discussed in this paper.

Advances in Technique and Technology

Carbon Coating

Since most food materials are not good conductors, frozen samples for X-ray analysis must be coated with a thin layer of conducting material whose X-ray energy peaks do not overlap with those of the elements of interest in the specimen and which does not significantly absorb the X-rays emerging from the surface of the specimen. Unless good quality uniform coatings can be obtained, charging may occur and it may then be difficult to obtain accurate analytical results because incident electrons will be deflected as they near the specimen surface and give rise to X-rays some distance from the area being analysed. Defects in the surface coating quickly become evident in those analyses which require that the electron beam dwells on each part of the specimen for several seconds, as described below for quantitative mapping.

The material most widely used for coating is carbon in the form of cord or rods which must be evaporated onto the surface of the specimen, usually by resistive heating. However, because most cryo-preparation units operate only at rotary pump pressures, i.e. 1.33 - .133 Pa, the mean free path of the evaporated carbon is relatively short and little of it is able to reach the surface of the specimen before the carbon source is exhausted. The carbon source must then be recharged and the evaporation process repeated (after de-gassing).

It has been claimed that this problem can be obviated by the use of the dedicated type of cryo-preparation unit which is permanently attached to the specimen chamber of the microscope. By opening the gate valve between the microscope column and work chamber of the cryo-preparation unit, the vacuum for carbon evaporation becomes equal to that of the microscope (1.33×10^{-3} - 10^{-4} Pa); when the gate valve is closed, it might be supposed that carbon can be evaporated onto the surface of the specimen at high efficiency. However, using a cryo-preparation unit fitted with a Penning gauge head it can be shown that during this operation the pressure in the work chamber rises to about 1.33×10^{-2} Pa by the time the carbon is hot enough to evaporate and by the end of evaporation it is almost back to the normal operating pressure of the rotary pump. In reality, high quality carbon films of adequate thickness cannot be obtained consistently in this way.

In order to ensure controlled carbon evaporation and to consistently produce even coatings of high quality, it is necessary to use a high vacuum system. In the present work this has been achieved with an EMscope SP2000 system by fitting a turbo-molecular pump (50 - 100 l/s) between the work chamber and the rotary pump of the cryo-preparation unit. Further details of this instrumentation have been given elsewhere (Brooker, 1990). Complete control over the thickness of the carbon film is highly desirable for reproducible results. This can be achieved using a quartz crystal oscillator of the type that has long been used to monitor the thickness of platinum/carbon films produced in freeze fracture units, with the sensor placed in a suitable place close to the carbon source and at the same level as the specimen position. The results presented in this study were obtained with the carbon source 40 mm from the specimen surface.

The minimum thickness of carbon that was found to give a coating suitable for electron beam dwell times of 300 - 500 ms was 5 nm but for quantitative mapping or point analysis in which longer dwell times were used, 12 - 20 nm of carbon was usually required to prevent charge accumulation on flat fracture surfaces.

Quantitative X-Ray Mapping

Digital X-ray mapping is a valuable technique which allows the spatial distribution of selected chemical elements to be determined within a given field of view. This type of mapping uses energy 'windows' corresponding in position to the elemental spectral energy peaks (whose range is set by the operator) to identify incoming X-rays from the specimen. For many applications this approach is perfectly adequate and presents few difficulties, but there are situations when this is not the case. If, for example, two elements of interest have partially overlapping X-ray energy ranges, it will not be possible by conventional digital mapping to distinguish the distribution of one from that of the other with certainty. In the case of food materials, this problem is likely to arise when potassium and calcium are being mapped in the same field of view. Because the potassium K_β peak partly overlaps the K_α peak of calcium, calcium maps will contain an indeterminable contribution from potassium K_β X-

rays and the results will not be reliable. In addition, if a given element is present at very low levels the results will not be accurate and/or the element may not be detected.

These problems may be overcome using the technique of quantitative mapping. In this study the mapping was performed using commercial software with the Link Analytical AN 10/85 X-ray spectrometer but other versions have also been used with a range of other biological materials (e.g. Fiori et al., 1988; Saubermann and Heyman, 1987).

This quantitative mapping technique identifies and evaluates elements at each pixel on the basis of standard spectra files derived from known standards in the same way as conventional quantitative 'ZAF' analysis. This means that elements whose spectral energy peaks completely overlap (e.g. sulphur and molybdenum) can be mapped separately and accurately and, of course, the same applies for the relatively simple case of potassium and calcium. A departure from normal digital elemental mapping is the ability to perform automatic background subtraction and to produce a map depicting the values of the FIT index (as in normal quantitative analysis, the FIT index is a measure of the fit of the stored profiles from the standards to the unknown spectrum from the specimen; for further information of FIT index, see Appendix) obtained at each pixel (Figs 1 & 2); the maximum pixel FIT index from this map is then displayed. The significance of this facility is that it allows areas of the map which are of doubtful accuracy, because of such factors as spectrometer drift or inaccurate standard peak profiles, to be immediately recognised. The FIT index should be as low as possible; a map with uniformly high FIT indices would indicate serious problems.

A good example of the use of the digital FIT index in this way can be seen in Figs 1 and 2. Focal concentrations of potassium in cotyledon cells of frozen and fractured sunflower seed can be seen in the X-ray map of Fig. 1; looking at the digital FIT indices from the same area (Fig. 2) it can be seen that the values of the FIT index are randomly and evenly distributed throughout the field and do not show changes in value in those areas which correspond to the high levels of potassium in Fig. 1. This means that the spectra from the potassium standard and the sunflower seed compare very well and this in turn strongly indicates that the digital values for potassium concentration constituting Fig. 1 have a high degree of reliability. This approach has been discussed in more detail by Statham (1988a).

A further advantage of this technique is that it permits the statistical fluctuations in the value of individual pixels to be recorded in the form of a standard deviation map. This provides a means of applying a rigorous statistical test to examine the likelihood of a given feature being real and not a product of doubtful statistics. In practice this means that a map may be displayed with a confidence level of one, two or three standard deviations, in the manner used by Brooker (1990) for particles of cocoa solids and milk protein in chocolate.

Further details of this powerful technique have been given by Statham (1988 a, b) so that it is necessary here only to point out that whereas the normal time for acquiring X-rays (dwell time) in quantitative point analysis is in the order of 100s, the corresponding time for mapping has been reduced to about 1.5 s (minimum)/pixel. Therefore, even for a map of low resolution (e.g. 64 x 64 pixels) the total time required to produce a map with electron beam dwell times of 2 - 3 s/pixel, may be many hours. As referred to above, this sort of exposure of the specimen to the electron beam is a good test of the quality of the carbon coating.

In the case of frozen food samples, this may mean that the temperature of the specimen must be maintained at liquid nitrogen temperature overnight if the map is to run to completion. The fitting of large dewars (e.g. 1 - 1.5 l capacity) to the cryo-SEM stage will allow the specimen to remain cold for several hours but the additional use of one of the commercially available automatic liquid nitrogen topping-up device is inevitable if longer unattended periods are required. The long acquisition times required by this analytical technique dictate that the sample is coated with a high quality carbon film.

Cryo-milling

It is always assumed that a sample ready for X-ray analysis has a reasonably flat surface and has been coated with a suitable conductor. Except for some specialised applications, fracturing is the normal method of preparing a clean surface for examination but it should always be remembered that this fracture plane is, by definition, the plane of greatest mechanical weakness. It is therefore pertinent to ask whether this method of preparation, even though it is now universally used in freeze fracturing studies, reveals structures that are representative of the sample as a whole. Furthermore, in this method of preparation there is very little or no control over the exact plane of fracturing. The operator may select the zone in which the fracture is to be initiated by applying the fracturing force in that area or by designing a special specimen holder (Wergin & Erbe, 1989) but the precise point at which fracturing will take place and its line of propagation cannot be predicted.

It is often desirable to fracture the sample more than once in order to examine the distribution of structures or chemical elements in several successive levels throughout the specimen. With certain favourable materials, such as oil in water emulsions, this can be done two or three times but for very brittle specimens (as in some seeds), the initial fracture usually shatters the whole structure and it is impossible to repeat the operation once the first plane of fracture has been coated and examined. Moreover, fracturing frequently results in the formation of an uneven surface, often with extremes of topography (Fig. 3), which is quite unsuitable for X-ray analysis. It is clear that this level of control over sample preparation leaves much to be desired. Indeed, Marshall (1988) has noted 'if fracture planes that were flat and reproducible could be easily obtained, some of the difficulties of analysing frozen-hydrated bulk samples (by quantitative X-ray microanalysis) would be

considerably reduced'.

One approach which overcomes all of these problems for many materials is cryo-milling. The principle is very simple; a frozen sample maintained at liquid nitrogen temperature is milled down to a desired level by a rotating diamond cutting tool to produce a perfectly flat surface ideal for coating with carbon and for X-ray analysis. The only commercially available instrument that can be used in this way is the Reichert-Jung Polycut E; this is a milling device that was originally developed for use at room temperature with very hard materials such as ceramics and metals.

During operation, the diamond rotates, the specimen is passed under it for milling and then the return stroke, with retraction, completes the cycle. The diamond mills for no more than half of each rotation. During the remainder of each rotation, it is lifted from the surface of the specimen to ensure that it does not touch part of the specimen surface that has already been milled. This is achieved by mounting the diamond milling head on a shaft that is at an angle of 2" (seconds) from vertical. The consequence is that the milled surface is not truely flat but the resulting undulations are so small that they cannot be detected by SEM. The thickness of the milled layer in any one cycle may be between 1 and 999 um and the total Z feed is 70 mm. The speed at which the sample is milled is continuously variable between 0.1 - 100 mm/s although for best results with the food systems that have been examined, the maximum milling rate should be no greater than 1 mm/s.

By installing a freezing stage in place of the conventional specimen holder, the same apparatus can be used to mill frozen food materials. The specimen is either frozen rapidly in a cryogen and attached to the freezing stage in a clamped specimen holder or embedded in Tissue Tek and frozen and then milled to the desired level in the specimen (Fig. 4). The temperature of the bulk samples during milling is continuously variable by controlling the amount of liquid nitrogen passing through the specimen stage; for the samples examined in this study the temperature for milling was maintained at $-165^{o}C$.

In order to keep the surface of the milling specimen free of frost, it is necessary to surround the apparatus with a plastic box through which dry nitrogen is passed and to fit a suitable interface into the side of the box to allow the specimen to be taken from the miller to the cryo-preparation unit for coating via a cryo-transfer device.

Once milling and carbon coating have been completed, it is possible to perform analysis and to study structural features under almost ideal conditions on a surface that may have less than 100 nm undulation (depending on the sample). For example, Figs 5 and 6 are of a carbon coated cocoa bean after it has been milled; they show the smooth appearance of the folded cotyledons and part of the endosperm. Because of their brittle fracture properties, cocoa beans are among the most difficult specimens to prepare with a flat surface for analysis and are therefore ideal samples with which to test the effectiveness of milling. Similar results were obtained in the course of this study with a variety of fat-rich foods, such as cheese and chocolate.

In spite of these results, two major questions arise from this method of specimen preparation, namely: (a) to what extent does frictional heating of the specimen surface take place during milling and (b) does cryo-milling cause any smearing of the structural and/or chemical components across the surface of the specimen ?

To date, there have been no accurate measurements of the temperature changes that take place at the surface of the specimen during milling. However, in foods containing some water, such as low fat spreads, the ice crystals at the surface of milled samples are of similar size to those found in the same material after freeze fracturing to the same level in the specimen by cryo-SEM. This suggests that if a temperature rise does occur during milling, it is not sufficient to promote the growth of ice crystals at the surface of the specimen. Furthermore, none of the food materials that have been cryo-milled to date have shown contamination of their surface by debris; this suggests that as material is milled from the surface, it is thrown to one side of the specimen by the rotating diamond.

The examination of the surface of a large number of different types of food materials after cryo-milling has failed to produce any morphological evidence of smearing. However, it would be possible for chemical components to be smeared across the milled surface without detectable morphological changes in structural components. For this reason, X-ray analysis has been used to examine the surface of milled samples for evidence of a re-distribution of the normal complement of elements. The cocoa bean is a suitable specimen to use for this purpose because the cotyledons contain groups of cells (probably corresponding to the pigment cells described by Winton & Winton (1939)) which preliminary studies have shown to possess high levels of potassium (and phosphorus). Since the surrounding cells are filled largely with fat (cocoa butter) and contain only a thin rim of cytoplasm with very low levels of these elements, any smearing of the focal concentrations of potassium would be clearly evident.

Thus, when quantitative X-ray maps of potassium distribution (at known confidence levels) are produced from the cotyledons of cocoa beans which have been cryo-milled and carbon coated (Fig. 7), the 'pigment' cells are clearly visible in groups (Fig. 8) as discrete focal concentrations of potassium. At higher magnifications individual 'pigment' cells can be seen in detail (Fig. 9) and the outlines of more numerous cells (probably containing fat) are visible as thin layers of cytoplasm containing barely visible levels of potassium. Examination of many areas of the cotyledons from several cocoa beans in this way has failed to produce any evidence of smearing effects that distort the normal distribution of chemical elements.

These results affirm the suitability of using cryo-milling to prepare smooth, flat surfaces of food materials prior to their examination by

cryo-SEM and X-ray microanalysis. Not only is mapping easier and more reliable, but the specimen surface is ideal for quantitative X-ray analysis also. Furthermore, milling provides total control over the selection of the plane in the specimen to be examined and it permits meaningful image analysis of features in a way that is not normally possible with SEM because of the general problem of variable topography.

Used together, the improvements in methodology referred to here help to ensure the reproducibility and reliabilty of results obtained from X-ray microanalysis of food systems when it is performed in conjunction with cryo-SEM.

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Appendix

The FIT Index in Quantitative X-ray Analysis:

In quantitative analysis, spectra from areas of interest in the specimen and known standards are initially processed by a method known as digital filtering. Deviations between the filtered standard profiles and filtered spectrum from the specimen are known as the residuals. The FIT index is calculated from the residuals and is equal to the average value of (r^2/σ^2), where r is the residual and σ the standard deviation for a given energy channel of the X-ray spectrum. The FIT index is thus a 'figure of merit' which describes the magnitude of the systematic errors in the fit which may be due to inaccurate peak profiles or spectrometer drift in relation to random statistical errors.

The validity of this approach has been argued extensively by Statham (1977). In the case of quantitative mapping, the maximum FIT index is the maximum value of this index for any of the pixels in any given field. Therefore, an indication of the reliability of the map can be obtained at a glance without referring to the FIT index of each and every pixel in the map.

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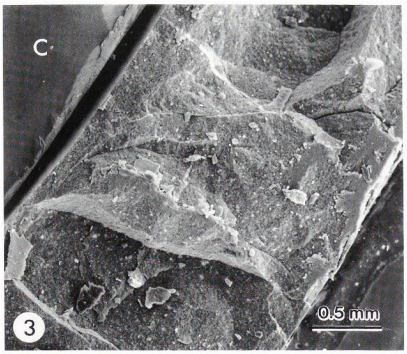
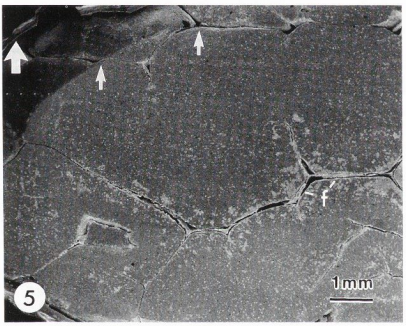
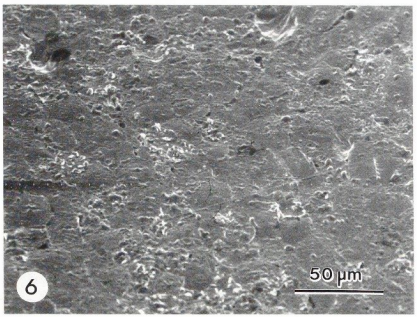
Discussion with Reviewers

D. Manning: Can freeze fracture be used first to identify morphological structures then finely mill the sample to expose a cross-section for X-ray microanalysis ? In this way, the morphological feature could be matched to the X-ray microanalysis data.

Author: There is no reason why this sort of manipulation cannot be done. Once the sample is frozen it is possible to transfer it from the milling device to the microscope and back again as many times as is required providing care is taken not to allow water vapour to condense on the specimen surface just before it is to be examined in the microscope.







All electron micrographs and X-ray maps were obtained using a Hitachi S-570 scanning electron microscope operating at 12kV and fitted with a cold stage. Cryo-SEM was performed using an EMscope SP2000 cryo-preparation unit; in all cases, the temperature of the microscope stage was maintained at - 165°C. Quantitative X-ray maps were produced using Link Analytical Remote Quantitation software; acquisition count rates were 1800 - 2000 counts/s (dead time 15%). All samples were frozen by plunging into nitrogen slush or liquid propane.

Fig. 1 Quantitative digital X-ray map of potassium distribution in sunflower cotyledon cells. Several cytoplasmic particles are rich in potassium. Dwell time 3 s. Resolution 64 x 64 pixels. Cryo-SEM.

Fig. 2 Digital FIT index map corresponding to the quantitative X-ray map of sunflower seed cotyledon cells shown in Fig. 1. Note that there is no increase in pixel intensity corresponding to the position of the potassium rich particles in Fig. 1. This, together with a maximum pixel FIT index of 2.1 (given by the software) indicates that the information contained in the quantitative maps is within acceptable limits of error. Resolution 64 x 64 pixels. Cryo-SEM.

Fig. 3 Frozen and fractured sample of a margarine showing the extremely variable topography. C = carbon sample holder. Cryo-SEM.

Fig. 4 The Reichert-Jung Polycut E fitted with a low temperature stage and milling a frozen sample. The rapidly rotating diamond head (H) appears slightly blurred in this picture; a frozen, and partially milled cocoa bean (B) is clearly visible embedded in a matrix of Tissue-Tek. The insulated liquid nitrogen supply line is also visible (S).

Fig. 5 Smooth surface of a milled cocoa bean showing the testa and folds (f) in the cotyledons. Part of the testa (at large arrow) has been removed after milling to show continuity of the cotyledon folds on the side of the bean with those seen on the milled surface (small arrows). White patches on the milled surface are areas of cells containing crystalline cocoa butter. Cryo-SEM.

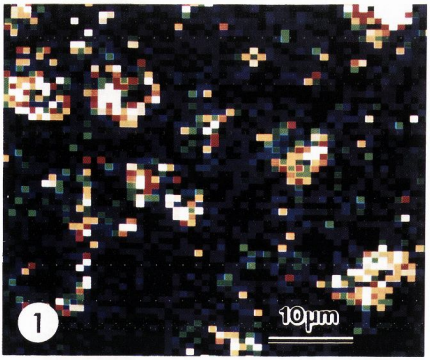
Fig. 6 The smooth surface of a cocoa bean cotyledon after milling. Cryo-SEM.

Figs 7 - 9 Quantitative digital X-ray maps of potassium distribution in cocoa beans. Dwell time 3s; 12kV. Resolution 128 x 128 pixels. Cryo-SEM.

Fig. 7 The testa contains relatively high levels of potassium; numerous 'pigment cells' in the cotyledon are rich in potassium and at this magnification appear as single or pairs of pixels.

Fig. 8 A group of potassium rich 'pigment cells' is surrounded by fat cells. The hole in some cells represents the position of the nucleus.

Fig. 9 A small area of cocoa bean cotyledon showing clearly defined, potassium rich 'pigment cells'. The fat cells (F) contain only low levels of potassium in a thin rim of cytoplasm.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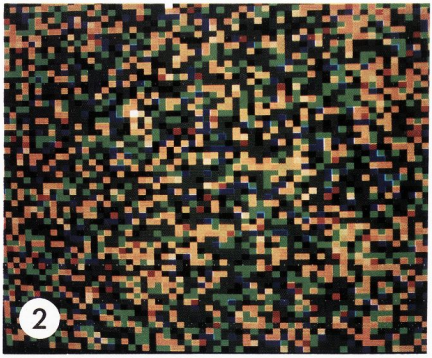
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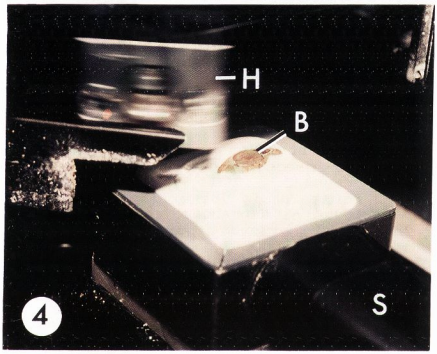
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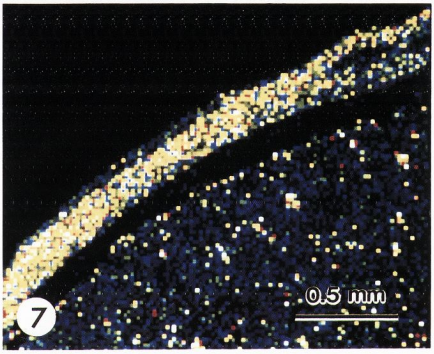
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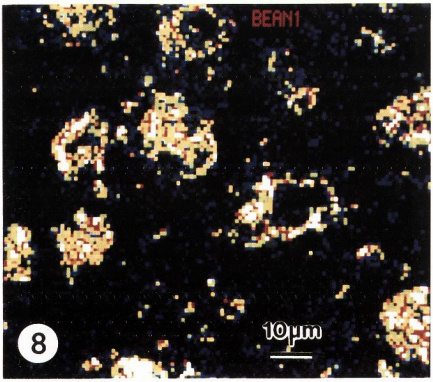
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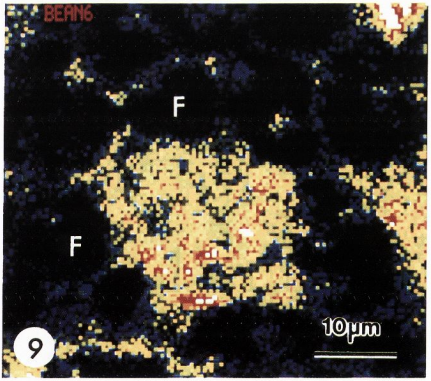
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10μm


INCREASING CONC.

D. Manning: Can this technique of X-ray microanalysis be used with food of higher moisture content; i.e. ice-cream ?
Author: Yes. Cryo-SEM is one of the few methods available for examining bulk samples of ice cream and other frozen foods with high water contents. Only when food materials are in this frozen state, is it feasible to perform valid X-ray micro-analysis.

G.M. Roomans: Your assessment of successful peak deconvolution in quantitative X-ray mapping may be too optimistic. You are right in pointing out that with the older method of X-ray mapping, overlap of elemental lines practically prevented meaningful mapping. However, in quantitative mapping, as in conventional quantitative point analysis, the accuracy of deconvolution depends on the counting statistics. In quantitative mapping, relatively short counting times per pixel are used, and counting statistics are not optimal. Have you carried out any quantitative assessment of the deconvolution procedure for, for example potassium $K\beta$ and calcium $K\alpha$?
Author: The counting statistics for quantitative mapping (typical counting time/pixel of 3 - 5 s) cannot be as good as those for quantitative point analysis (typical counting time 100 s) because the total number of X-rays collected and counted is relatively small. This is why quantitative mapping must incorporate software to determine the statistical confidence limits of the results obtained. It is this that provides the quantitative assessment of the deconvolution procedure for partially or completely overlapping elements. In practice, the procedure has been found to be extremely accurate and reliable.

J.C.G. Blonk: In Fig. 6 light features are present. Is this an indication of discontinuities in the carbon film ? Is there an indication for melting of fat crystals during mapping ?
Author: Crystals of cocoa butter on the surface of frozen samples do not melt even when the electron beam dwells on them for some minutes. It is difficult to identify the light areas in Fig. 6 with any certainty but it is possible that they represent minute discontinuities in the carbon film as you suggest.

J.C.G. Blonk: What is the accelerating voltage that you use in the mappings ? If it is 15kV, did you ever observe sub-surface charging which is not unlikely to occur in these non-conducting samples?
Author: The voltage used for the materials examined in this paper was 12kV but voltages up to 15kV have been used in the past with equal success. Even at 15kV charging does not occur if the carbon film is of high enough quality. However, I appreciate that many workers have great trouble depositing a carbon film onto frozen samples sufficent for this purpose. If the vacuum is good enough and the distance between specimen and carbon source is not too great (e.g. < 60 mm) it is difficult to understand why this should be.